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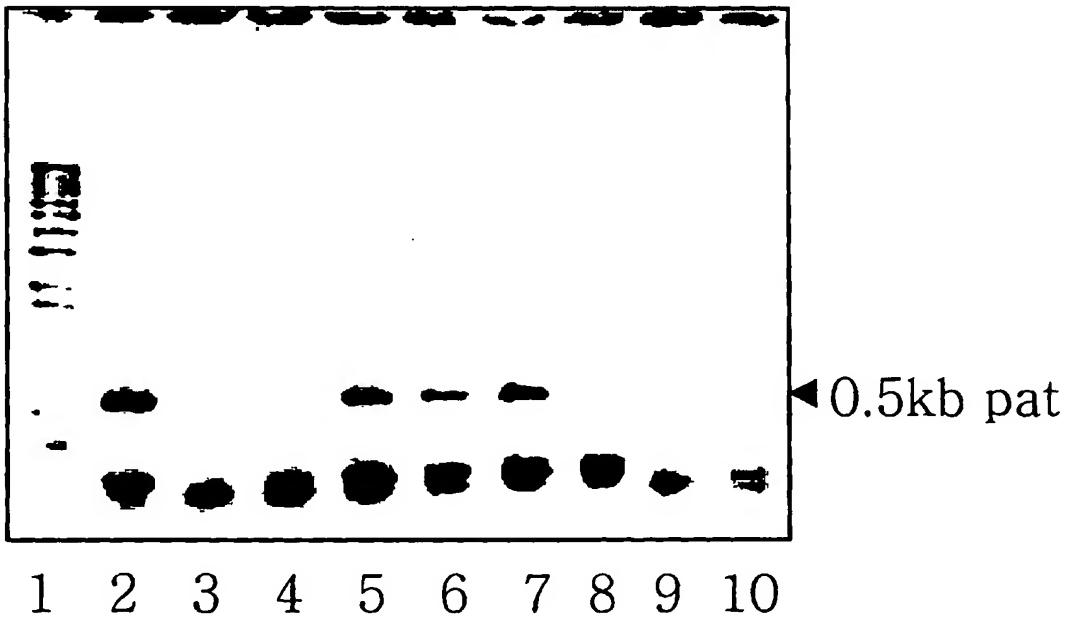
(54) Title: METHOD FOR PREPARING TRANSFORMED CUCUMIS SATIVUS



(57) Abstract: The present invention relates to a method of preparing a transformed Cucumis sativus using Acrobacterium tumefaciens, more particularly, to a method for preparing a transformed Cucumis sativus, which comprises the steps of: (a) inoculating a cotyledon from Cucumis sativus with Agrobacterium tumefaciens harboring a suitable vector; (b) placing the inoculated cotyledon on a medium containing BAP (6-benzylaminopurine) and NAA (a-naphthalene acetic acid) and culturing the inoculated cotyledon to obtain regenerated shoots; and (c) culturing the regenerated shoots on a rooting medium to obtain the transformed Cucumis sativus.

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METHOD FOR PREPARING TRANSFORMED CUCUMIS SATIVUS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a method for preparing a transformed *Curcumas sativa*, more particularly, relates to a method of preparing a transformed *Cucumis sativus* using *Acrobacterium tumefaciens* and a transformed *Cucumis sativus* prepared therefrom.

Cucumbers (*Cucumis sativus* L.) belonging to Cucurbitaleae are classified into a type of rising outdoors consumed primarily in the Europe and a type of raising greenhouse consumed primarily in the United Kingdom. Cucumbers cultivated in the Asia can be classified into southern part types and northern part types. In Korea, Cucumbers are estimated to have appeared about 1,500 years ago. Cucumbers may be used as fresh or processed vegetable and have been commonly employed as raw material in cosmetics such as facial lotion and pack, contributing to income of farmhouses.

Though the cultivation area of raising outdoors of cucumber in Korea has been on an decreasing trend, the total area under cultivation has become to be increased due to a rapid increase of cultivation area using facilities. As a result of a spread of cultivation using facilities, a modernization of the facilities, an improvement of a cultivar of cucumber and a development of

cultivation technologies, the total yield of cucumber in 1995 reached twice that in 1980.

In the cultivation of cucumbers, the prime consideration matter is damage from various diseases. It has been reported that the diseases found in cucumbers are caused by fungi, bacteria and virus. The occurrence of diseases by fungi and bacteria may be reduced through the prevention with agricultural chemicals and/or continuous management, but the damage by virus cannot be avoided yet. In addition, the damage by noxious insects may be considerable and the treatment with insecticides is recommended as a sole method of prevention. Especially, in parallel with increasing cultivation area using facilities, the damages by noxious insects of various kinds has been on the increase, but the reliable solution thereto has not been provided yet, which is due primarily to problems including that a period of 5-10 years is required in breeding a cultivar having desirable character. Thus, as one of solutions to such problems, the genetic engineering technology capable of introducing a variety of characters into plant has been already recommended, rendering a breeding period shortened.

Up to now, many attempts have been made for regeneration and a transformation of cucumber. For example, it has been reported that the cucumbers regenerated have been obtained from hypocotyls (Rajasekaran et. al., Flower formation in vitro by hypocotyl explants of cucumber

(*Cucumis sativus* L.) Ann-Bot. London:Academic Press., 52(3):417-420(1983)), leaves (Chee and Tricoli, Somatic embryogenesis and plant regeneration from cell suspension cultures of *Cucumis sativus* L. *Plant-Cell-Rep.* Berlin, 7(4):274-277(1988)), cotyledons (Cade et. al. Organogenesis and embryogenesis from cucumber(*Cucumis sativus* L.) cotyledon-derived callus. *HortScience* 22:1130(1987); Gambley,-R.L.; Dodd,-W.A. Effect of hypocotyl length on morphogenesis of explants of cucumber(Cucumis sativus L.) in vitro. 19(2):165-169(1992)), pollen (Lazarte and Sasser, Asexual embryogenesis and plantlet development in anther culture of Cucumis sativus L. Cucumber, regeneration, tissue culture. *Hortsci. American Society for Horticultural Science.* 17(1):88(1982)), protoplasts (Jia,-S.R. et al. Embryogenesis and plant regeneration from cotyledon protoplast culture of cucumber(Cucumis sativus L.). *J-Plant-Physiol.* 124(5):393-398(1986); Colijn-Hooymans et al.. Competence for regeneration of cucumber cotyledons is restricted to specific developmental stages. *Plant-cell,-tissue-organ-cult.* 39(4):211-217(1994)), and the like.

However, according to these method developed up to now, there are remained problems such as low regeneration rate, abnormal growth of regenerated plants and earlier blooming and ageing *in vitro* culture (Ziv and Gadasi, Enhanced embryogenesis and plant regeneration from cucumber (Cucumis sativus L.) callus by activated charcoal in

solid/liquid double-layer cultures. *Plant-Sci.* 47(2):115-122(1986); Gambley,-R.L.; Dodd,-W.A. Effect of hypocotyl length on morphogenesis of explants of cucumber (Cucumis sativus L.) in vitro. 19(2):165-169(1992); Kim J.W. et al., Plant regeneration through organogenesis and somatic embryogenesis of Cucumber (*Cucumis sativus* L.). *Korean J. Plant Tissue Culture*, 25(2):125-129(1998)).

Thus, there remain many things to be improved as to regeneration and transformation of cucumbers.

Throughout this application, various publications are referenced and citations are provided in parentheses. The disclosure of these publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

Under such situation, the present inventors have made intensive research to be from the shortcomings of the conventional transformation methods for *Cucumis sativus* and as a result, we have developed a successful method to ensure preparation of a transformed *Cucumis sativus* with higher reproducibility.

Accordingly, it is an object of this invention to provide a method for preparing a transformed *Cucumis sativus* using *Agrobacterium tumefaciens*.

It is another object of this invention to provide a transformed *Cucumis sativus*.

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjunction with the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows regenerated cotyledons from *Cucumis sativus* transformed according to this invention;

Fig. 2 represents a genetic map of binary vector pRD320 used in this invention;

Fig. 3 demonstrates comparison between transformant of this invention and non-transformant;

Fig. 4 demonstrates the effect on rooting depending on agar concentration in rooting medium;

Fig. 5a shows the results of GUS analysis confirming the occurrence of transformation in *Cucumis sativus*; and

Fig. 5b shows the results of PCR elucidating transformed *Cucumis sativus* according to this invention.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of this invention, there is provided a method for preparing a transformed *Cucumis sativus*, which comprises the steps of: (a) inoculating a cotyledon from *Cucumis sativus* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a

genome of a cell from *Cucumis sativus* and contains the following sequences: (i) a replication origin operable in the cell from *Cucumis sativus*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis sativus*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence; (b) placing the inoculated cotyledon on a medium containing BAP (6-benzylaminopurine) and NAA (α-naphthalene acetic acid) and culturing the inoculated cotyledon to obtain regenerated shoots; and (c) culturing the regenerated shoots on a rooting medium to obtain the transformed *Cucumis sativus*.

The present inventors have made extensive research with 5 cultivars of *Cucumis sativus* developed in Korea, and finally developed methods for regeneration with higher frequency and transformation with *Agrobacterium tumefaciens* in more efficient manner, as exemplified in Examples below:

The present invention will be described in more detail as follows:

I. Preparation of Starting Material for Transformation

The preferred explants for transformation includes leaf, stem and petiole, but not limited to. The explants may be obtained from several plant organs and most preferably from seed. It is preferred that the seed is sterilized

with sterilizing agent such as chlorine and chlorides (e.g., sodium hypochloride) before use.

II. Seed Germination

According to a preferred embodiment of this invention, the medium for seed germination comprises nutrient basal medium such as B5, LS, N6 and White's, energy source and vitamins, but not limited to. Sugars are useful as energy source and sucrose is the most preferable. It is preferred that vitamins for seed germination include nicotine, thiamine and pyridoxine. In addition, the medium for seed germination in this invention may further contain MES (2-(N-Morpholino) ethanesulfonic acid Monohydrate) as buffering agent for pH change and agar as solid support. The medium is unlikely to contain plant growth regulators.

III. Preparation of Plant Tissue for Transformation

In this invention, the explant for transformation includes any tissue derived from seed germinated. It is preferred to use cotyledon and hypocotyl and the most preferred is cotyledon. It is advantageous to remove growth point completely from cotyledon as explant and to use explant in whole not in dissected one.

IV. Inoculation with *Agrobacterium tumefaciens*

Transformation of cells derived from *Cucumis sativus* is carried out with *Agrobacterium tumefaciens* harboring Ti

plasmid (Depicker, A. et al., Plant cell transformation by *Agrobacterium* plasmids. In Genetic Engineering of Plants, Plenum Press, New York (1983)). More preferably, binary vector system such as pBin19, pRD400 and pRD320 is used for transformation (An, G. et al., Binary vectors" In Plant Gene Res. Manual, Martinus Nijhoff Publisher, New York(1986)).

The binary vector useful in this invention carries: (i) a replication origin operable in the cell from *Cucumis sativus*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis sativus*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence. In addition to this, it is preferred that the vector carries antibiotics-resistance gene as selective marker, e.g. carbenicllin, kanamycin, spectinomycin and hygromcin. The vector may alternatively further carry a gene coding for reporter molecule (for example, luciferase and β-glucuronidase). Examples of the promoter used in the binary vector include but not limited to Cauliflower Mosaic Virus 35S promoter, 1′ promoter, 2′ promoter and promoter nopaline synthetase (nos) promoter. The structural gene in the present vector may be determined depending on traits of interest. Exemplified structural gene may include but not limited to genes for herbicide resistance (e.g. glyphosate, sulfonylurea), viral resistance, vermin resistance (e.g., Bt gene), resistance to environmental extremes (e.g.

draught, high or low temperature, high salt conc.), improvement in qualities (e.g. increasing sugar content, retardation of ripening), exogenous protein production useful as drug (EGF, antigen or antibody to various diseases, insulin) or cosmetic raw material (e.g. albumin, antibiotic peptide).

Inoculation of the explant with *Agrobacterium tumefaciens* involves procedures known in the art. For example, the most preferred explant, cotyledon with removed growth point is immersed in medium of *Agrobacterium tumefaciens* to coculture, thereby inoculating the cotyledon with *Agrobacterium tumefaciens*. Preferably, acetosyringone is employed in the coculturing to promote infection of *Agrobacterium tumefaciens* into explant cell.

V. Regeneration

It is necessary that explant tissue, which is transformed with *Agrobacterium tumefaciens*, be regenerated in regeneration medium with strictly controlled ingredients and quantities thereof. The regeneration medium of this invention may contain nutrient basal medium such as MS, B5, LS, N6 and White's, energy source and vitamins, but not limited to. Sugars are useful as energy source and sucrose is the most preferable. It is preferred that vitamins in regeneration medium include nicotine, thiamine and pyridoxine. In addition, the regeneration

medium may further contain MES (2-(N-Morpholino) ethanesulfonic acid Monohydrate) as buffering agent for pH change and agar as solid support.

The medium must contain plant growth regulators. Cytokinin as plant growth regulator may include but not limited to 6-benzylaminopurine (BAP), kinetin, zeatin and isopentyladenosine and BAP is the most preferable cytokinin. Furthermore, the regeneration medium contains, as essential ingredient, auxin such as NAA (α-naphthalene acetic acid), indole acetic acid and (2,4-dichlorophenoxy) acetic acid, and the most preferable is NAA.

Preferably, the amount of BAP in the regeneration medium, ranges from 1 to 4 mg/l, more preferably from 1.5 to 2.5 mg/l and most preferably 2.0 mg/l. If the amount is less than 1 mg/l or exceeds 4 mg/l, the regeneration rate tends to drop sharply.

The preferable amount of NAA in the regeneration medium, is in the range of from 0.001 to 0.08 mg/l, more preferably from 0.005 to 0.03 mg/l and most preferably 0.01 mg/l. If the amount is less than 0.001 mg/l or exceeds 0.08 mg/l, the regeneration rate is very likely to drop sharply.

The regeneration medium of this invention removes the necessity of $CuSO_4$ or casein hydrolysate which is generally used in the art for regeneration of plants belonging to Cucurbitaleae. Surprisingly, the medium of the invention exhibits improved regeneration rate and

ability in regenerated shoot formation of *Cucumis sativus* without $CuSO_4$ or casein hydrates, which clearly demonstrates excellency of the medium.

According to a preferred embodiment of this invention, the medium further contains antibiotics (e.g. carbenicllin, kanamycin, spectinomycin or hygromcin) for selection of transformed explant. In the case of using kanamycin as selective marker, 70-150 mg/l is a preferred amount. If the amount is less than 70 mg/l, the final transformation rate becomes 0 while the regeneration rate is higher; and if exceeding 150 mg/l, the regeneration rate is very likely to dive dramatically.

Most preferably, the cotyledon placing is performed by insertion on the regeneration medium. Unlikely to leaf section, placing cotyledon at full length on the medium results in negligible regeneration and callus formation.

Culturing according to the conditions described above allows successfully a regeneration of shoots through callus formation from the transformed explant of *Cucumis sativus* on the medium.

VI. Rooting

The transformed *Cucumis sativus* plantlet is finally produced on rooting medium by rooting of regenerated shoots. The rooting medium of this invention may contain nutrient basal medium such as MS, B5, LS, N6 and White's, energy source and vitamins, but not limited to. Sugars are

useful as energy source and sucrose is the most preferable. It is preferred that vitamins in the rooting medium include nicotine, thiamine and pyridoxine. In addition, the rooting medium may further contain MES (2-(N-Morpholino) ethanesulfonic acid Monohydrate) as buffering agent for pH change and agar as solid support.

As plant growth regulator, auxin is predominantly employed in the rooting medium. The auxin useful includes NAA, indole acetic acid and (2,4-dichlorophenoxy) acetic acid, and the most preferable is NAA. Preferably, the amount of NAA in the rooting medium is in the range of from 0.001 to 0.08 mg/l. If the amount is less than 0.001 mg/l, the period necessary for rooting is relatively longer and thinner/longer roots without root hairs are frequently induced. If the amount exceeds 0.08 mg/l, the stem in contact with medium tends to be corpulent leading to no occurrence of rooting.

Alternatively, where the rooting medium contains agar as solid support, 0.2-0.7%(w/v) is preferable amount. If the amount is less than 0.2%, the support function is largely diminished; if exceeding 0.7%, the elongation of roots is remarkably retarded, thicker/shorter roots are formed and rooting is likely to occur out of medium.

VII. Confirmation of Transformation

The transformed *Cucumis sativus* produced according to the present invention may be confirmed using procedures

known in the art. For example, using DNA sample from tissue of transformed *Cucumis sativus*, PCR is carried out to reveal exogenous gene incorporated into a genome of *Cucumis sativus* transformed. Alternatively, Northern or Southern Blotting may be performed for confirming the transformation as described in Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.(1989). Where the vector harbored in *Agrobacterium tumefaciens* contains a gene encoding β-glucuronidase, the tissue from cotyledon regenerated is immersed in substrate solution such as X-gluc (5-Bromo-4-Chloro-3-Indole-β-D-Glucuronic Acid) so that colorimetric reaction may be observed to confirm the occurrence of transformation.

According to the most preferred embodiment of this invention, there is provided a method for preparing a transformed *Cucumis sativus*, which comprises the steps of: (a) inoculating an intact cotyledon from *Cucumis sativus* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell from *Cucumis sativus* and contains the following sequences: (i) a replication origin operable in the cell from *Cucumis sativus*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis sativus*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence; (b) placing the

inoculated cotyledon on a medium containing 1.5-2.5 mg/l of BAP (6-benzylaminopurine) and 0.005-0.03 mg/l of NAA (α-naphthalene acetic acid) by insert and culturing the inoculated cotyledon to obtain regenerated shoots; and (c) culturing the regenerated shoots to obtain the transformed *Cucumis sativus* on a rooting medium containing 0.005-0.03 mg/l of NAA and 0.2%-0.7% agar.

In another aspect of the present invention, there is provided a transformed *Cucumis sativus* prepared by the methods of this invention described above.

The method of this invention, which is developed for producing a transformed *Cucumis sativus*, as exemplified and demonstrated in Examples below, exhibits much higher transformation and regeneration efficiency, leading to production of transformed *Cucumis sativus* having desirable traits with higher reproducibility.

The following specific examples are intended to be illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

EXAMPLE 1 Preparing of Explants

5 cultivars of *Cucumis sativus* (JanghyongNakhap, BagbongDadaki, IbchuNakhap, SamnamCheongjang and KangryeokSamcheok), which have been developed in Korea,

were employed in transformation experiments. Seeds from 5 cultivars were sterilized for 15 min. in 5% NaOCl solution and washed 3 times for 15 min. with sterilized DW. The sterilized seeds were placed on germination media containing 1/2 MSMS (Murashige & Skoog medium including Minimal Salts), 2% sucrose and 0.6% agar and then cultured to germinate seed for 4 days at 25±1℃ under dark condition. Thereafter, seedlings without true leaf were selected and cotyledon and hypocotyl were taken therefrom.

EXAMPLE 2: Construction of Medium Composition for Regeneration

To construct a suitable medium composition for regeneration of cotyledon or hypocotyl, 10 types of media were prepared with a combination of cytokinin (BAP, zeatin and kinetin) and auxin (NAA). See Table 1. The basal media containing MSB5 (Murashige & Skoog medium including Gamborg B5 vitamins), 500 mg/ℓ of MES(2-(N-Morpholino) ethanesulfonic acid Monohydrate), 3% sucrose and 0.6% agar were employed for regeneration.

TABLE 1

	Cytokinin (mg/l)			NAA (mg/l)
	BAP	Kinetin	Zeatin	
Medium 1	2.00	-	-	-
Medium 2	2.00	-	-	0.01
Medium 3	2.00	-	-	0.10
Medium 4	4.00	-	-	0.01

Table 1 (continued)				
Medium 5	4.00	-	-	0.10
Medium 6	-	2.70	-	5.00
Medium 7	-	3.00	-	0.20
Medium 8	-	5.00	-	1.00
Medium 9	-	-	1.00	0.10
Medium 10	-	-	2.00	0.20

Thereafter, each of 20 individuals was placed on each medium above and cultured for 30 days at 26±1℃ and 8,000 lux under the condition of 16 hrs/8 hrs (light/dark), followed by examination of regeneration rate and average number of regenerated shoots. The regeneration rate was calculated from percentage of ratio of the number of regenerated section to total number of section placed and the average number of regenerated shoot was calculated from percentage of ratio of the number of regenerated shoot to the number of regenerated section. The results are summarized in Table 2.

TABLE 2

Medium	Cultivar of *Cucumis sativus*									
	I[1]		J[2]		K[3]		B[4]		S[5]	
	Reg[6]	Shoot #[7]	Reg	Shoot #	Reg	Shoot #	Reg	Shoot #	Reg	Shoot #
1	0	0	0	0	0	0	0	0	0	0
2	30	1.2	23	1.1	15	1.5	90	2.4	25	1.5

Table 2 (continued)

3	25	1.0	20	1.2	13	1.2	65	3.2	15	1.5
4	25	1.0	13	1.0	5	1.1	85	1.3	15	1.0
5	20	1.2	10	1.1	10	1.1	25	1.2	0	1.2
6	13	1.6	15	1.2	25	1.2	0	0	13	1.2
7	15	1.1	10	1.4	10	1.1	10	1.0	0	0
8	-	-	-	-	-	-	15	1.1	15	1.2
9	25	1.2	25	1.2	15	1.5	75	1.9	35	1.5
10	15	1.4	10	1.1	13	1.2	65	2.1	45	1.3

[1)]IbchuNakhap, [2)]JanghyongNakhap, [3)]KangryeokSamcheok, [4)]BagbongDadaki, [5)]SamnamCheongjang, [6)]Registration rate (%), and [7)]average number of shoot regenerated

As shown in Table 2, the regeneration rate and the ability in shoot formation were exhibited in the wide range of 0-90% and 1.0-3.1, respectively, depending on cultivar used. Among 5 cultivars, BagbongDadaki showed the most excellent regeneration rate and ability in shoot formation. In addition, compared to results from zeatin and kinetin, it was elucidated that BAP is the most suitable in view of regeneration rate and ability in shoot formation. At 2 mg/l concentration, BAP was found to accomplish higher regeneration rate and ability in shoot formation, while at more than 4 mg/l BAP to decrease regeneration rate. NAA, at relatively low conc. (0.01 mg/l), was found to give rise to higher regeneration rate and ability in shoot formation compared to the results from no addition and high conc.(0.1 mg/l). In the media

containing both 2 mg/l of BAP and 0.01 mg/l of NAA, the highest regeneration rate were exhibited, the average number of shoot regenerated was found to be much larger (2.5 per section) and the period necessary for regeneration was found to be excellent (approx. 20 days). All results above were obtained from cotyledon. As to hypocotyl, the regeneration rate was much lower, shoots were rarely regenerated, and callus formed were finally dead.

Cotyledons, which were in dark cultivation for 4-5 days, were found to form callus at the region in contact with media, which appeared from about 7 days of culture in the regeneration media; and with the lapse of 15 days, the regenerated shoots emerged from corpulent callus, finally leading to a multitude of shoots (See Fig. 1). In Fig. 1, panels A and B represent shoots regenerated from cotyledons at 10 and 20 days, respectively from placing on media.

As revealed from the results, as explant for transformation of *Cucumis sativus*, cotyledon is the most preferable, the medium optimal for transformation contains BAP (2 mg/l) as cytokinin and NAA (0.01 mg/l) as auxin.

EXAMPLE 3: Evaluation of Regeneration Rate Depending on Explant

The cotyledons cut off at 2 mm above from seedlings were placed on the medium 2 in Table 1 which was

determined optimal and the regeneration rate and average shoot number thereof were examined in the same manner as Example 2.

From the results, placing with the cotyledon in whole gave rise to higher regeneration rate and average shoot number. Therefore, for regeneration, the cotyledon in whole is preferred than one with upper part removed.

EXAMPLE 4: Evaluation of Regeneration Rate Depending on Placing Method

In an effort to evaluate the effect of placing method on regeneration rate, the cotyledons in whole, which exhibited the highest reproduction rate in Example 3, were placed on media 2 of Example 2 in insertion or placing at full length, followed by determining the regeneration rate in the same manner as Example 2.

From the results, it can be understood that unlikely to leaf section, placing cotyledon at full length on the medium results in negligible regeneration as well as poor formation of callus. Contrary to regeneration with leaf section, insertion on medium is the most efficient placing method for regeneration of cotyledon from *Cucumis sativus*.

EXAMPLE 5: Examination of Effects of Trace Elements on Regeneration Rate

$CuSO_4$ and casein hydrolysates which are generally used in the art for enhancing a regeneration of plants

belonging to Cucurbitaleae. Media 2 in Example 2 containing various concentrations of $CuSO_4$ and casein hydrolysates were prepared and the regeneration rate was determined in the same manner as Example 2.

It was revealed that $CuSO_4$ and casein hydrolysates could not enhance regeneration of cotyledons from *Cucumis sativus*. Therefore, it can be known that the regeneration method according to this invention may avoid the use of $CuSO_4$ and casein hydrolysates in regeneration media.

EXAMPLE 6: Construction of Conditions for Transformation

In order to construct the optimal conditions for transformation, the optimal procedures, periods and temperature for coculturing were examined by use of BagbongDadaki showing the most excellent regeneration rate and MSB5 (Murashige & Skoog medium including Gamborg B5 vitamins) media containing 2 mg/l of BAP and 0.01 mg/l of NAA.

Firstly, the seeds from the cultivar BagbongDadaki were germinated in the same manner as Example 1 and cotyledons were taken. *Agrobacterium tumefaciens* GV3101 (Mp90) (*Plant-cell-rep.* 15(11):799-803(1996)) harboring *p*RD320 vector (Omirulleh,-S. et al., Activity of a chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. *Plant. Mol. Biol. Int. J. Mol. Biol. Biochem. Genet. Eng.*

21(3): 415-428(1993)) with *gus::npt*Ⅱ fusion gene and *pat* (phosphinithricin acetyl transferase) gene was cultured for 18 hrs in super broth (37 g/ℓ Brain heart infusion broth(Difco), 0.2% sucrose, pH 5.6) and the cotyledons were immersed into the media followed by mixing for 10 min.

Then, in coculturing medium (MSB5 medium with 2 mg/l of BAP and 0.01 mg/l of NAA), the mixture was cocultured for 2 days at 25℃ under conditions for light culture. Alternatively, after light cultivation, coculturing with *Agrobacterium tumefaciens* and cotyledons was carried out for 4 days at 4℃. Following coculturing, without performing clean up step, the cotyledons inoculated with *Agrobacterium tumefaciens* were cultured for 4 weeks at 26±1℃ and 8,000 lux under 16 hrs/8 hrs (day/night) condition in media containing 2 mg/l BAP, 0.01 mg/l NAA, 500 mg/l carbenicillin and 100 mg/l kanamycin in addition to MS-B5, 0.5 g/l MES, 3% sucrose and 0.4% phytagel.

The regenerated shoots were transferred to the rooting medium containing NAA (0. 0.01 or 0.1 mg/l), 100 mg/l kanamycin and agar (0.4%, 0.6% and 0.8%) and further cultured at 26±1℃ and 8,000 lux under 16 hrs/8 hrs (day/night) condition to obtain final transformants with roots, which were determined as Example 7.

TABLE 3

	Coculture conditions	Cotyledon #[3]	Reg.[1]		Transform[2]	
			No.[4]	Rate (%)	No.[5]	Rate (%)
1st Exp.	RT[6]	200	40	20.0	10	5.0
	LT[7]	200	12	6.0	3	1.5
2nd Exp.	RT	100	20	21.0	4	4.0
	LT	100	7	7.0	2	2.0
Tota l	RT	300	61	20.3	14	4.7
	LT	300	19	6.3	5	1.7

[1]regeneration, [2]transformation, [3]number of cotyledons used in experiment, [4]number of regenerated cotyledons, [5]number of transformant, [6]coculture at room temperature, and [7]coculture at low temperature (4℃)

As shown in Table 3, the coculture for 2 days at room temperature (25℃) represented much higher regeneration and transformation rate than the coculture for 4 days at lower temperature (4℃). Furthermore, the cotyledons cocultured at lower temperature were not regenerated and became necrotic with etiolation.

Fig. 3 demonstrates the difference between successful transformant and non-transformant which was found not to generate root and become necrotic with etiolation. In Fig. 3, panels A and B represent transformant and non-transformant, respectively.

TABLE 4

	Conc. Of kanamycin(mg/l)	Cotyledon #[1)]	Reg.[2)]		Transform[3)]	
			No.[4)]	Rate (%)	No.[5)]	Rate (%)
1st Exp.	50	40	35	87.5	-	-
	100	40	12	30.0	2	5.0
	200	40	2	5.0	0	0.0
2nd Exp.	100	100	31	31.0	14	3.1
	200	100	3	3.0	0	0.0

[1)]number of cotyledons used in experiment [2)]regeneration, [3)]transformation, [4)]number of regenerated cotyledons, and [5)]number of transformant

As indicated in Table 4, in the case of using the selection medium containing 50 mg/l of kanamycin, the regeneration rate appeared high but the transformation rate was found to be about 0. Moreover, the selection medium containing 100 mg/l of kanamycin showed about 30% of regeneration rate and about 3% of transformation rate, and that with 200 mg/l of kanamycin revealed about 3% of regeneration rate and about 0% of transformation rate. Consequently, it can be understood that the preferred concentration of kanamycin is about 100 mg/l in the selection medium.

In the rooting medium, as NAA concentration became higher, the roots generated were found to be thicker and shorter, and the period required for rooting to be shorter. In the rooting medium containing 0.1 mg/l of NAA, it was

observed that the stem in contact with medium tended to be corpulent leading to no occurrence of rooting. In the case of no treatment with NAA, the period necessary for rooting was relatively longer and thinner/longer roots without root hairs were frequently induced. Therefore, it can be known that the preferred concentration of NAA in rooting medium is about 0.01 mg/l.

Moreover, the rooting medium with relatively low agar (0.4% and 0.6%) was likely to result in normal roots with feasibility; but in the case of the rooting medium containing above 0.8% agar, the elongation of roots was remarkably retarded, thicker/shorter roots were formed and rooting was likely to occur out of medium (See Fig. 4). In Fig. 4, panels A and B represent rooting patterns in media with 0.6% and 0.8% agar, respectively.

EXAMPLE 7: Confirmation of Transformant

The transformants produced in Example 6 were confirmed as follows:

Example 7-1: GUS Analysis

To measure activity of β-glucuronidase, the tissues from cotyledons regenerated were immersed in X-gluc (5-Bromo-4-Chloro-3-Indole-β-D-Glucuronic Acid) solution and incubated for 24 hrs at 37℃. The developed colorimetric reaction (blue) was observed with naked eye to confirm the occurrence of transformation (Fig. 5a).

Example 7-2: PCR Analysis

For template in PCR, the isolation of genomic DNA from plant was performed using the method described by Edwards K., et al. (*Nucleic Acids Research*, 19: 1349(1991)). The primers for PCR were designed to have complementary sequence to *pat* gene of the vector in *Agrobacterium tumefaciens*: forward primer, 5'-AGA CCA GTT GAG ATT AGG CCA G-3' and reverse primer, 5'-GCC TCA TGC AAC CTA ACA GA-3'. The PCR using Taq polymerase was performed in such a manner that pre-denaturation at 96℃ for 2 min. and denaturation at 94℃ for 1 min. were done consecutively, and total 35 cycles were done in which each cycle is composed of denaturation step at 94℃ for 1 min., annealing at 55℃ for 1 min. and extension step at 72℃ for 2 min., followed by final extension at 72℃ for 10 min. The PCR product was subject to electrophoresis on 1.0% agarose gel (Fig. 5b). In Fig. 5b, lanes M, 1, 2 and 3 show 1 kb ladder, PCR product of plasmid carrying *pat* gene as positive control, PCR product of genomic DNA from non-transformed *Cucumis sativus* and PCR product of genomic DNA from transformed *Cucumis sativus*, respectively. As shown in Fig. 5b, the PCR product of genomic DNA from *Cucumis sativus* transformed according to this invention, exhibited 0.5 kb DNA band corresponding to *pat* gene, which demonstrates the successful transformation.

A number of reports, which are directed to regeneration

of *Cucumis sativus*, have been made to develop novel *Cucumis sativus* cultivars with desirable traits. Notwithstanding this, the critical problems, for example, low regeneration rate and viability, abnormal growth, earlier blooming and ageing *in vitro* culture, have not been solved. The present invention can be free from the disadvantages and problems of the conventional method described previously. That is, referring to the results from Examples 6 and 7, it can be fully understood that the present invention provides *Cucumis sativus* with about 20-30% of regeneration rate and about 4% of transformation rate by use of cotyledon as explant and suitable regeneration and transformation conditions, which are superior to that of the conventional methods.

In conclusion, according to the present invention, novel *Cucumis sativus* with desirable traits can be obtained with higher regeneration and transformation rate.

What is claimed is:

1. A method for preparing a transformed *Cucumis sativus*, which comprises the steps of:

(a) inoculating a cotyledon from *Cucumis sativus* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell from *Cucumis sativus* and contains the following sequences:

(i) a replication origin operable in the cell from *Cucumis sativus*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis sativus*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence,

(b) placing the inoculated cotyledon on a medium containing BAP (6-benzylaminopurine) and NAA (α-naphthalene acetic acid) and culturing the inoculated cotyledon to obtain regenerated shoots; and

(c) culturing the regenerated shoots on a rooting medium to obtain the transformed *Cucumis sativus*.

2. The method according to claim 1, wherein the cotyledon of step (a) is in whole not dissected one.

3. The method according to claim 1, wherein the step of placing is performed in such a manner that the total parts of the cotyledon are inserted into the medium layer.

4. The method according to claim 1, wherein an amount of BAP in step (b) is 1-4 mg/l.

5. The method according to claim 4, wherein the amount of BAP is 1.5-2.5 mg/l.

6. The method according to claim 1, wherein an amount of NAA in step (b) is 0.001-0.08 mg/l.

7. The method according to claim 6, wherein the amount of NAA is 0.005-0.03 mg/l.

8. The method according to claim 1, wherein an amount of NAA in the rooting medium is 0.001-0.08 mg/l.

9. The method according to claim 8, wherein the amount of NAA in the rooting medium is 0.005-0.03 mg/l.

10. The method according to claim 1, wherein the rooting medium further comprises 0.2%-0.7% agar.

11. A method for preparing a transformed *Cucumis sativus*, which comprises the steps of:

(a) inoculating an cotyledon in whole from *Cucumis sativus* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting

into a genome of a cell from *Cucumis sativus* and contains the following sequences:

(i) a replication origin operable in the cell from *Cucumis sativus*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis sativus*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence,

(b) placing the inoculated cotyledon on a medium containing 1.5-2.5 mg/l of BAP (6-benzylaminopurine) and 0.005-0.03 mg/l of NAA (α-naphthalene acetic acid) by insert and culturing the inoculated cotyledon to obtain regenerated shoots; and

(c) culturing the regenerated shoots to obtain the transformed *Cucumis sativus* on a rooting medium containing 0.005-0.03 mg/l of NAA and 0.2%-0.7% agar.

12. A transformed *Cucumis sativus* prepared by the method according to any one of claims 1-11.

Fig. 1

Fig. 2

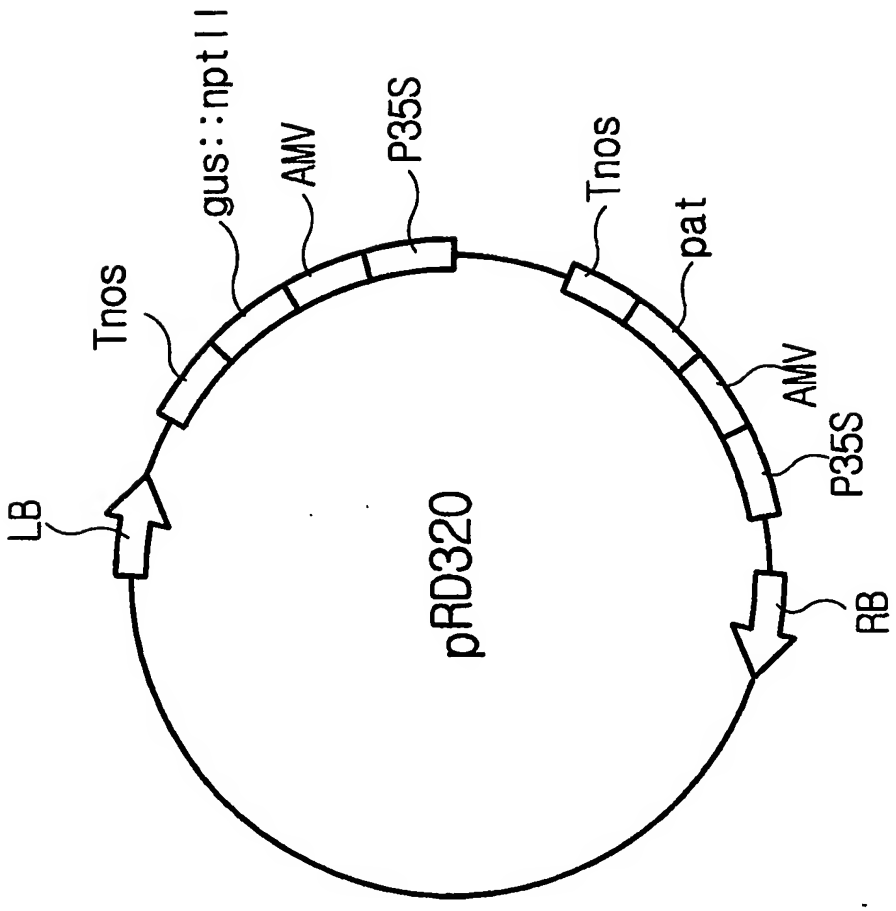


Fig. 3

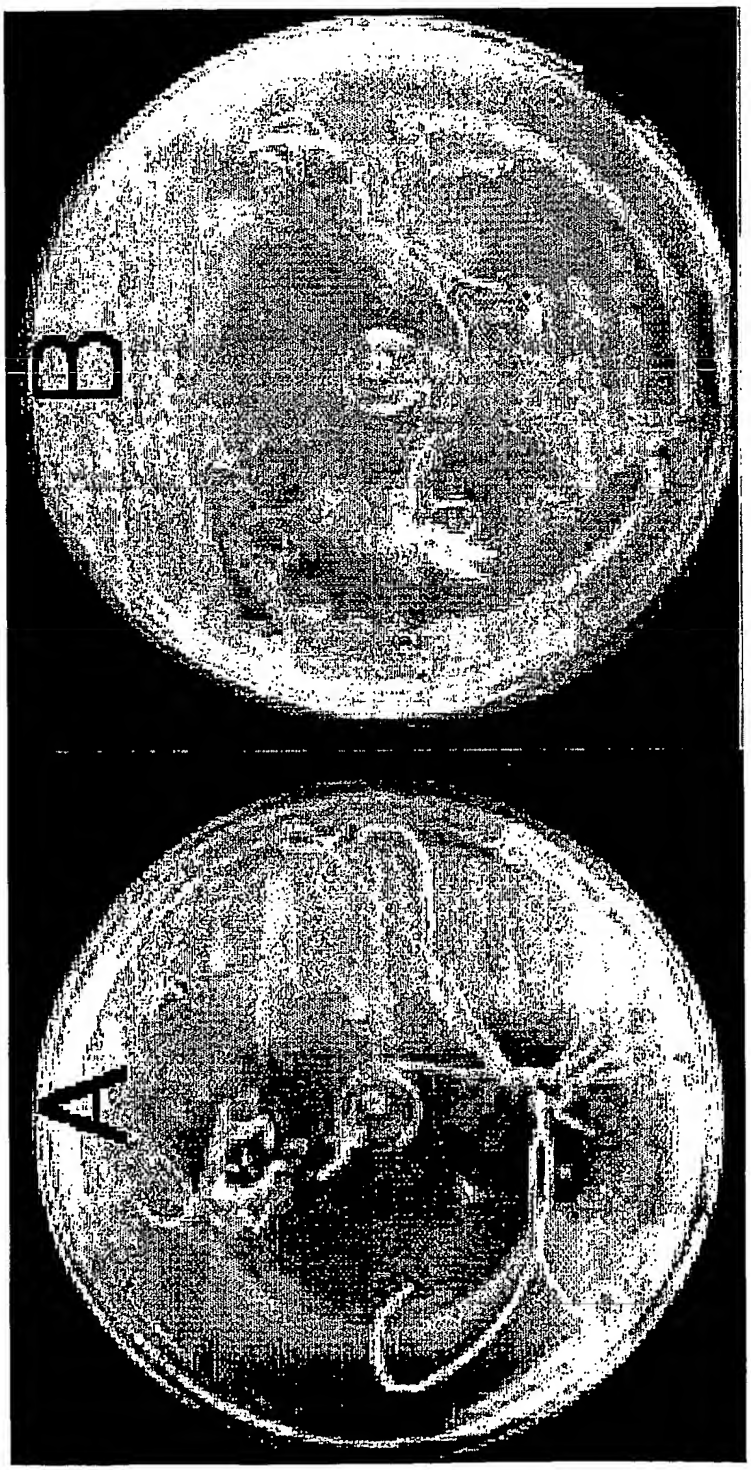
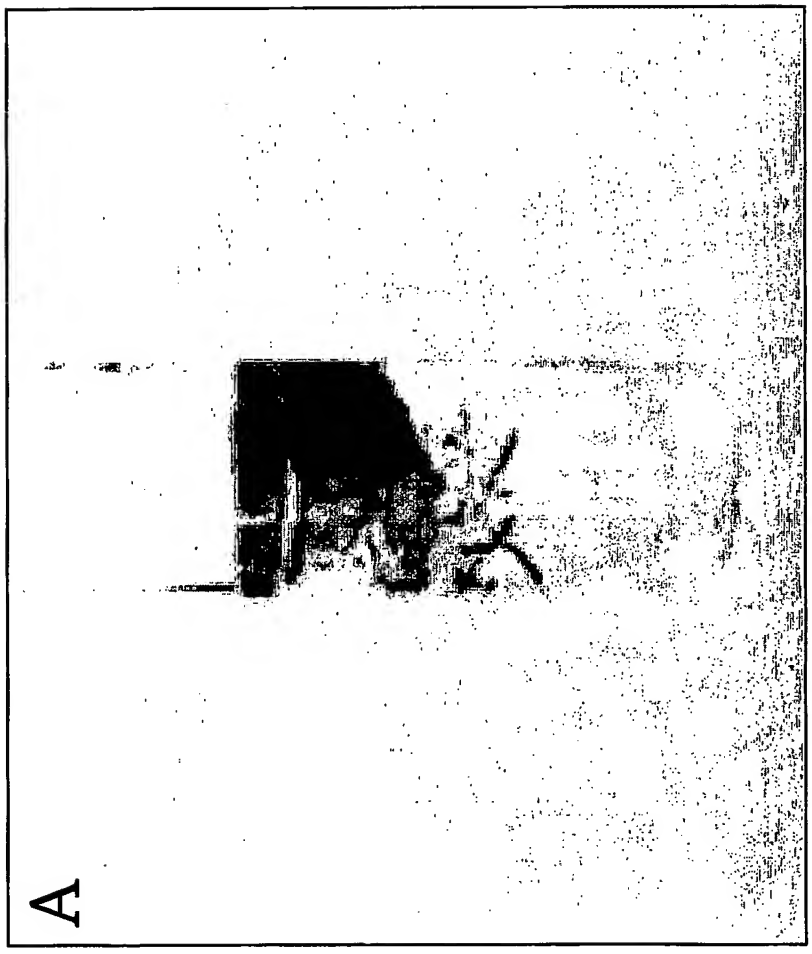


Fig. 4

Fig. 5a



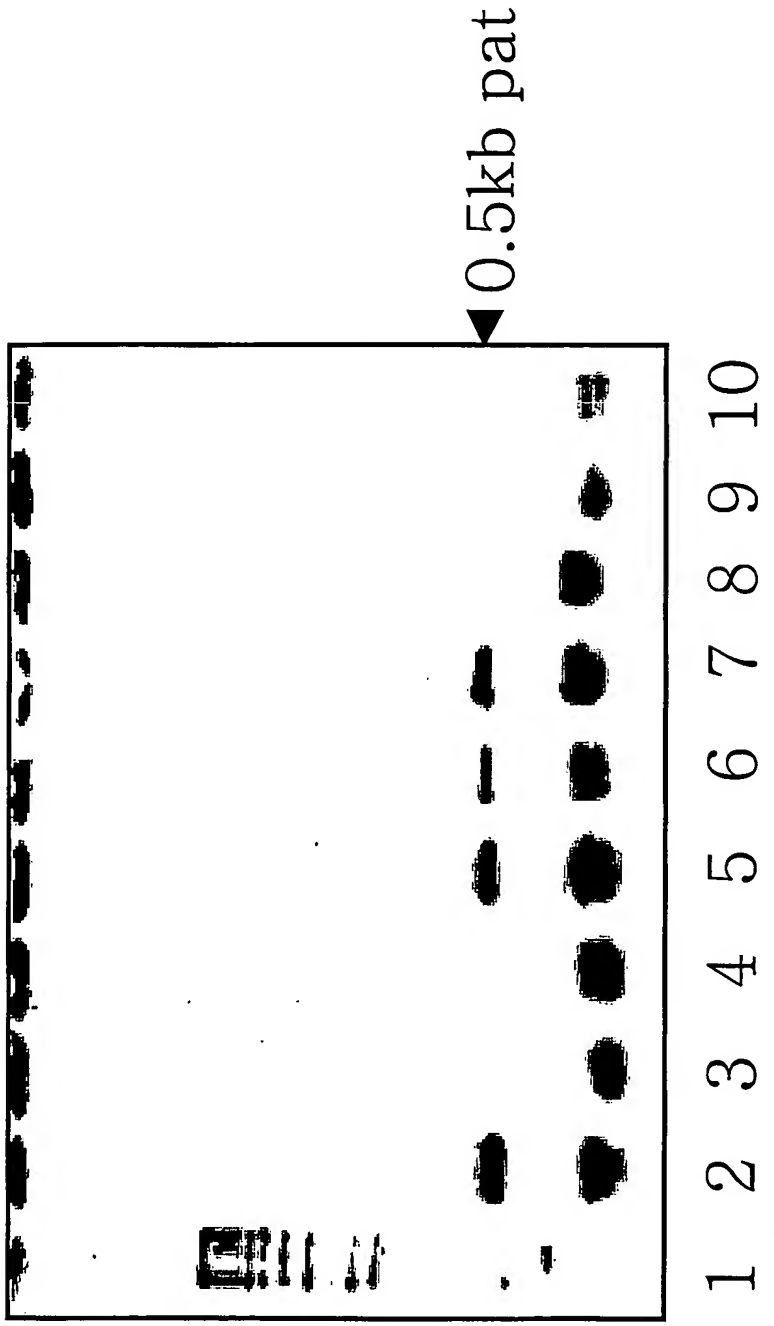


Fig. 5b

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CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C12N 15/82, 15/29, A01H 4/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C12N 15/82, 15/29, A01H 4/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 88/02399 A1 (THE PLANT CELL RESEARCH INSTITUTE, INC) 7 April 1988 (07.04.88) *claims 1-4,7.*	1,11,12
A	WO 90/03725 A1 (THE UPJOHN COMPANY) 19 April 1990 (19.04.90) *pages 10-13,18,19; claims.*	1,11,12
A	EP 0952224 A2 (KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY) 27 October 1999 (27.10.99) *claims 1,2,7-9.*	1-3,11,12
A	JP 05 276844 A (MITSUBISHI CORP et al.) 26 October 1993 (26.10.93) (abstract) WPI [online]. London, U.K.: Derwent Publications, Ltd. [retrieved on 12-11-2002]. DW199347, AN: 1993-373516 [47] *abstract.*	1,11,12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 November 2002 (12.11.2002)	4 December 2002 (04.12.2002)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer MOSSER R. Telephone No. 1/53424/437

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				KR	B1	270910	01-11-2000
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				KR	A	00014481	15-03-2000
JP	A2	5276844	26-10-1993			none	
WO	A1	8802399	07-04-1988	AU	A1	81026/87	21-04-1988
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				EP	A2	262971	06-04-1988
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				JP	T2	2500406	15-02-1990
WO	A1	9003725	19-04-1990	AU	A1	44182/89	01-05-1990
				CN	A	1041784	02-05-1990
				EP	A1	438475	31-07-1991
				JP	T2	4501354	12-03-1992